DISCUSSION FORUM

Failure to adequately use positive control data leads to poor quality mouse lymphoma data assessments

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The mouse lymphoma L5178Y/TK<sup>+/−</sup>-3.7.2C mutagenesis assay (MLA) is widely used to identify chemicals that are capable of inducing mutational damage (Mitchell et al., 1997). Mutants detected in the assay form a bimodal distribution based on colony size. More than two decades of research demonstrate that both small and large colony thymidine-resistant (TFT) colonies are ‘true mutants’ and that the assay detects the broad spectrum of genetic damage known to be involved in the etiology of cancer (Moore-Brown et al., 1981; Moore et al., 1985b; Applegate et al., 1990; Hozier et al., 1992). Because the assay detects mutations that act through a variety of mechanisms, the MLA is specifically recommended by regulatory agencies for inclusion in hazard identification screening for environmental chemicals and pharmaceuticals (Dearfield et al., 1991; ICH3, 1996).

Because of the ability of the MLA to detect both point mutations and chromosomal mutations, it would be expected that some chemicals would be positive in the MLA but not in the Salmonella assay, which only detects chemicals capable of inducing point mutations. In addition, it would be expected that the MLA would yield mutant frequencies (including the spontaneous background mutant frequency) far higher than those characteristic of other gene mutation assays, such as hypoxanthine guanine phosphoribosyl transferase deficiency and ouabain resistance, which by their nature are unable to detect the same broad spectrum of mutational events (Moore et al., 1989). In fact, mutant frequencies >1000×10<sup>−6</sup> have been induced by a number of chemicals (Clive et al., 1979; Moore et al., 1989; Backer et al., 1990; Moore and Doerr, 1990).

A properly conducted MLA includes the demonstration that both small and large colony mutants have been optimally quantitated. This is accomplished by the appropriate use of the positive control. Chemicals that have been successfully used as positive controls (in the absence of exogenous activation) include methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS) and hycanthone. Mutant frequencies for these chemicals (and colony sizing information) were published in the early to mid 1980s (Moore and Clive, 1982; Moore et al., 1985a). Despite the presence in the early mouse lymphoma literature of acceptable mutagenicity data for these positive control chemicals, there have been several important papers published that contain positive control data that are totally unacceptable.

The US EPA Gene-Tox MLA Workgroup (Mitchell et al., 1997) found it impossible to make definitive determinations for a large proportion of the published MLA data. In fact, 23% of the chemicals in the published literature had insufficient data or unacceptable data, thus making it impossible to determine if the chemical was or was not mutagenic. One of the major reasons for this problem was the inadequacy of the positive control data. This inadequacy was particularly prevalent in the data generated by one of the contract laboratories used by the NTP; data by which the NTP evaluated the utility of the MLA (Tennant et al., 1987).

Suboptimal (and unacceptable) positive controls are readily identified by evaluating the magnitude of the positive control mutant frequencies. Using the published MMS- and/or EMS-induced mutant frequencies from laboratories that optimize for small colony detection as the acceptable standard, it is readily apparent that some laboratories are very inefficient in quantitating the total possible tk mutant frequency. For instance, Figure 1 shows the distribution of MMS-induced mutant frequencies found in 47 separate determinations in our EPA laboratory and 247 experiments reported by McGregor et al. (1988a,b, 1991a,b) from the Inveresk Research Institute. For this comparison the background mutant frequency was subtracted from the total mutant frequency. For the Inveresk laboratory, duplicate culture values are presented as the mean.

It is clear from this analysis that the MMS-induced mutant frequency is significantly different in the two laboratories. The EPA laboratory (Figure 1, column A) had no cultures in which the MMS-induced mutant frequency was <400×10<sup>−6</sup> and only three in which the frequency was <600×10<sup>−6</sup>. On the other hand, in only 19 of 247 determinations did the Inveresk MMS-induced mutant frequency exceed 400×10<sup>−6</sup>, with a maximum observed value of 616×10<sup>−6</sup> (Figure 1, column C). The majority of values (162 of 247) were <200×10<sup>−6</sup> (McGregor et al., 1988a,b, 1991a,b).

The induced large colony tk mutant frequency (from the EPA laboratory) is plotted in Figure 1, column B. The values seen for the EPA large colony MMS-induced mutant frequency are very similar to the total mutant frequency seen in the Inveresk laboratory, leading one to speculate that this laboratory might be quantitating only the large colony tk mutant frequency. Further evidence for this hypothesis is seen in Figure 2, where the ethyl acrylate total, large and small colony tk mutant frequencies obtained by the EPA laboratory are plotted along-

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261
side the total tk mutant frequency reported by McGregor et al. (1988b). As with the MMS data, it appears that McGregor and co-workers evaluated only the large colony mutant frequency. This inefficient mutant frequency quantitation causes serious problems when data from such laboratories are analyzed and compared with other laboratories, particularly when simple + or – calls (rather than the actual data) are presented and used to obtain conclusions.

When evaluating data from the MLA, it is important to remember that the assay, when properly performed, can give a very broad range of induced mutant frequencies. The significance of a positive test chemical response should depend not only on its ‘positive’ determination but also on the magnitude of the response. If small colonies are not optimally detected, it is impossible to evaluate adequately the significance of the detected mutant frequency. Many people who see MLA data think that mutant frequencies of 200–300×10⁻⁶ are strong responses. As discussed above, this is not true. Mutant frequencies of the order of 3000–4000×10⁻⁶ are possible (Backer et al., 1990). Thus, when using this assay, it is possible to distinguish very strong responses from those that are weak or marginally positive. Any statistical method will find the former
responses to be positive; the weak or marginal 'gray area' responses may be found positive by some statistical methods but not by others. If, however, the assay is conducted under conditions that do not optimally detect the small colony mutant frequency, the information inherent in the assay is lost.

The distinction between clear positive and weak or marginal responses becomes important when data from this assay are compared with data obtained from other test systems when a 'weight of evidence' approach is used in evaluating a battery of test data. For instance, a weak or marginal response in the MLA (particularly when only obtained at highly toxic concentrations and/or high concentrations of the test chemical) may not be particularly significant in light of other information, including how the chemical is metabolized. On the other hand, a high mutant frequency induced at concentrations causing low toxicity and/or relatively low concentrations of chemical is cause for concern. It should be noted that these comments are equally true for the data obtained using in vitro cytogenetic analyses.

The purpose of the positive control in any mutagenicity assay is to demonstrate convincingly that the system was working properly at the time a particular test compound was evaluated. It is not enough for the positive control to give a 'positive' response. The positive control must give an appropriate response. It is clear from the data presented by McGregor and co-workers that the MLA was not working optimally in that laboratory at the time the data were collected for the NTP evaluation of short-term tests.

While our analysis of the data reported by McGregor et al. (1988a,b, 1991a,b) indicates an inefficiency in small colony mutant quantitation, the reason(s) for this problem is not clear. There are, of course, two aspects to the adequate quantitation of small colony mutants; the proper growth of the mutants and the optimal counting of such mutants. The evaluation and correction of such problems must be accomplished by each individual laboratory.

Because of the importance of the data obtained from properly performed MLAs, we strongly urge every laboratory that conducts the assay to: (i) compare their positive control data with that found in the literature, published by laboratories known to optimize for small colony detection; (ii) modify assay conditions to improve the growth and counting of small colony mutants; (iii) perform colony sizing for all positive controls; (iv) publish colony sizing histograms for representative positive control cultures. Unless these steps are taken by all laboratories performing and publishing data from the assay, the quality of much of the data in the literature will continue to be poor and the utility will be limited.

Finally, it is important to note that when only data that meet certain adequacy criteria are used (Clive et al., 1995; Sofuni et al., 1997) the Gene-Tox Committee concluded, in contrast to the conclusions drawn by the National Toxicology Program (Tennent et al., 1987), that the MLA is highly predictive of the rodent cancer bioassay (Mitchell et al., 1997). This information, coupled with all of the known mechanistic information about the MLA, strengthens the argument for the inclusion of the assay in the required screening batteries for pharmaceutical and environmental chemical safety evaluations and for regulatory submissions.

References


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